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| (54) Title: PRENATAL GENETIC DETERMINATION (57) Abstract The present invention relates to a method of prenatal genetic testing by detecting the presence or absence of foetal nucleic acid in a blood sample obtained from the maternal circulation. In particular the method is applicable to foetal sex determination, prenatal diagnosis of genetic disorders and predispositions, sex linked or otherwise and assessment of placental permeability to foetal cells under various physiological or pathological conditions. | | |

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PRENATAL GENETIC DETERMINATION

The present invention relates to a method of genetic testing and in particular to a method of
5 detecting the presence or absence of a given genotype or genotypes in the genome of a foetus by testing a sample during pregnancy from the maternal circulation.

The placenta is thought to be an effective barrier against the transfer of cells and a number of
10 attempts to detect foetal nucleated cells in the maternal circulation during pregnancy have proved unsuccessful or at least unreliable. Thus for example, by examining meaphase chromosomes Walknowska J. et. al. (Practical and theoretical implications of
15 foetal/maternal lymphocyte transfer. Lancet 1969; i:1119-1122) demonstrated the existence of cells with a probable male karyotype in the peripheral circulation of mothers carrying a male foetus. This method, however, is very time-consuming and it has been
20 shown that such cells are found in the blood of normal, non-pregnant women. Walknowska J. et. al. (Practical and theoretical implications of foetal/maternal lymphocyte transfer. Lancet 1969; i:1119-1122). These studies were followed by others
25 employing quinacrine staining to detect circulating cells possessing Y-bodies from mothers bearing male foetuses (Schroder J, De La Chapelle A. Foetal lymphocytes in the maternal blood. Blood 1972; 39: 153-62 and Grosset L. et. al. Antenatal foetal sex
30 determination from maternal blood during early pregnancy. Am J. Obstet Gynecol 1974; 120: 60-63). These studies suffer from several drawbacks including non-uniform criteria for Y-body detection between laboratories, (Schroder J. Transplacental passage of

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blood cells. J. Med. Genet 1975; 12: 230-42) the occurrence of false-positives (Schroder J. De Al Chapelle A. Fetal lymphocytes in the maternal blood. "Blood 1972; 29: 153-62 and Grosset L. *et. al.*

5 Antenatal foetal sex determination from maternal blood during early pregnancy". Am. J. Obstet Gynecol 1974; 120: 60-63) and the failure of others to obtain the same results. (Zimmerman A. Schmickel R. "Fluorescent bodies in maternal circulation". Lancet 1971;

10 i:1305). With the development of flow cytometry Herzenberg *et. al.* (Herzenberg L. A. *et. al.* Detection and enrichment by fluorescence-activated cell sorting. Proc. Natl. Acad. Sci. (USA) 1979; 76: 1453-55) detected cells consistent with a foetal

15 origin by immunogenetic and cytogenetic criteria from peripheral maternal blood. By combining flow cytometry and monoclonal antibody technology Covone *et. al.* showed cells reacting with a monoclonal antibody H315 could be detected in peripheral blood

20 from pregnant women. (Covone A. E. *et. al.* Trophoblast cells in peripheral blood from pregnant women. "Lancet 1984; ii: 841-43). This antibody identified a glycoprotein that is expressed on the surface of human syncytiotrophoblasts (Johnson P. M. *et. al.* Human

25 trophoblast-specific surface antigen identified using monoclonal antibodies. Am. J. Reprod Immunol 1981; 1: 246-54). Subsequent investigations, however, have shown that most H315-positive cells present in

30 maternal circulation do not contain Y chromosome-derived DNA when the foetus is male and H315-negative cells have been shown to adsorb H315 antigen *in vitro*. (Covone A. E. *et. al.* Analysis of peripheral maternal blood samples for the presence of placenta-derived cells using Y-specific probes and McAb H315. Prenatal

35 Diagnosis 1988; 8: 591-607). These observations thus

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cast doubt on the significance of the earlier results.

The advent of the polymerase chain reaction (PCR) (Saiki R. K. et. al. Enzymatic amplification of Beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985; 230: 1350-54 and Saiki R. K. et. al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988; 239: 487-91) appeared to offer a promising alternative to the above approaches, as the exquisite sensitivity of the procedure is ideal for detecting the rare foetal cell amongst numerous maternal cells. A previous attempt, however, was unsuccessful in detecting circulating male foetal cells using PCR (see Adinolfi M. et. al. Gene amplification to detect foetal nucleated cells in pregnant women. Lancet 1989; ii: 328-329). Indeed the authors state that even if a few foetal lymphocytes, expressing class I and II major histocompatibility antigens cross the placental barrier, they are probably rapidly removed from the circulation (see Adinolfi M. The immunosuppressive role of alphafoetoprotein and the transfer of lymphocytes across the placenta: two controversial issues in the maternofoetal relationship. In: Adinolfi M. et. al. eds. Paediatric research: a genetic approach. London: Spastics International Medical Publications, 1982: 183-96). The authors further state that syncytiotrophoblastic cellular elements, shed into the uterine veins, seem to be

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trapped in the lung. (Covone A. E. et. al. Analysis of peripheral maternal blood samples for the presence of placenta derived cells using Y-specific probes and McAb H315-Prenatal Diagnosis 1988; 8: 591-607).

- 5 Finally the authors conclude that the present methods available for detecting foetal cells are not yet sensitive enough to allow non-invasive prenatal diagnosis.

- Schwinger et. al. (Am. J. Hum Genet
10 1989; 45:A268) attempted to identify male cells in blood from women bearing a male foetus. They attempted to amplify a 154bp repeat sequence from the Y chromosome using PCR. No specific signal was found.

- Most recently Yeoh S. C. et. al. (Detecting
15 foetal cells in maternal circulation, Lancet 1989; ii: 869-870) have suggested using HLA differences between mother and foetus as a means of identifying foetal leucocytes, but they conclude that their method is unlikely to be applicable to prenatal diagnosis since
20 foetal HLA types cannot be known in advance.

- The present invention is based upon the discovery of a method for detecting the presence or absence of a given genotype(s) (as hereinafter defined) in the genome of a foetus by testing a sample
25 obtained during pregnancy from the maternal circulation comprising foetal nucleic acid.

- According to one feature of the present invention there is provided a method for detecting the presence or absence of a given genotype (as
30 hereinafter defined) in the genome of a foetus, which comprises subjecting foetal nucleic acid obtained from the maternal circulation to amplification, in the absence of nucleic acid foreign to the foetus and to the mother, such foreign nucleic acid being capable of
35 amplification under the amplification conditions

- 5 -

employed, wherein at least two rounds of amplification are effected and whereby the presence or absence of a given genotype (as hereinafter defined) may be detected in the genome of the foetus.

5 The expression "genotype" as used herein means the genetic constitution of a cell which is responsible for or contributory to a recognisable phenotype. The expression "genotype" thus encompasses for example, genetic sequences characteristic of male
10 sex (Y chromosome) and genetic variants responsible for or contributory to disease, for example genetic disorders such as cystic fibrosis and the thalassaemias as well as predisposition to disease such as coronary heart disease and cancers.

15 It is important that the foetal nucleic acid subjected to amplification is not contaminated with foreign nucleic acid which foreign nucleic acid is capable of amplification under the amplification conditions employed. Thus for example where
20 amplification is effected by use of the polymerase chain reaction and appropriate primers are employed for the amplification of foetal nucleic acid it is irrelevant that foreign nucleic acid may be present which is incapable of amplification in the presence of
25 the aforementioned primers, but it is important that foreign nucleic acid which is capable of amplification in the presence of such primers is absent. Foreign nucleic acid which should be absent from the material to be amplified would be foreign in the sense that it
30 would be foreign to both the foetus and to the mother. The presence of such foreign nucleic acid in a sample to be amplified may give rise to erroneous results.

 The target nucleic acid sequence may be any sequence which is specific to the foetus. The
35 sequence may be present in multiple copies but the

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present invention can also be applied to single copy foetal sequences allowing for prenatal diagnosis of single gene disorders.

For purposes of foetal sex determination the target sequence may be any Y-chromosome specific nucleic acid sequence. The sequence may be present in multiple copies as illustrated in Example 1 below or as a single copy sequence as shown in Example 2. If the mother tested is bearing a male foetus the target sequence will be amplified to give a positive result, a female foetus will result in no DNA amplification.

Whilst whole cells may be used, in the method of the present invention it is preferred that extracted nucleic acid, preferably DNA be used. Whilst we do not wish to be bound by theoretical considerations it is believed that, at least in respect of nucleic acids comprising repetitive sequences; the use of extracted DNA rather than whole cells, may also increase sensitivity since in Example 1 referred to hereinafter the Y-specific sequence chosen is present as a tandem array of 800-5000 subunits (see Nakahori Y. *et. al.* "A human Y-chromosome specific repeated DNA family (DYZ1)" consists of a tandem array of pentanucleotides, "Nucleic Acids Res. 1986; 14, 7569-7580) which is broken up into much smaller subunits and dispersed into solution by the extraction procedure. Moreover, in respect of nucleic acids comprising repetitive sequences, it may be advantageous to effect partial fragmentation of foetal nucleic acid, prior to amplification, for example by vortexing, in order to shear long strands of nucleic acid. It is also advantageous that the nucleic acid from the rare foetal cells be mixed thoroughly with the maternal

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nucleic acid and such mixing may for example also be effected by vortexing.

According to the present invention the foetal nucleic acid is subjected to at least two rounds of amplification in order to increase the sensitivity of the method. It is advantageous however that no more than two rounds of amplification are employed since further rounds of amplification compound the risk of detection of contamination of the foetal nucleic acid with foreign nucleic acid. In this regard in relation to the polymerase chain reaction (as discussed below), each round of amplification will consist of multiple cycles of heating and cooling and the terms "round" and "cycle" are used in this context throughout the specification.

Amplification may be effected according to the method described by Klappe K. et. al. in J. Mol. Biol. (1971, 56, 341-361 (see page 360) and now commonly referred to as the polymerase chain reaction (PCR). This method (PCR) is also described in U.S. Patents Nos. 4683195 and 4683202. It will be appreciated however that amplification may also be effected by other techniques such as for example by the use of Q-beta replicase as described in PCT Patent Publication WO87/06270 and in Biotechnology Vol. 6 October 1988, by the use of the transcription based nucleic acid amplification of Siska Corporation as described in PCT Patent Publication WO88/10315, the method for isothermal amplification of nucleic acids described by Guatelli et. al. described in Proc. Natl. Acad. Sci., (1990, 87, 1874-1878), or by the use of linear amplification. In this connection the expression "linear amplification" is used herein

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to refer to amplification using a single primer for each diagnostic portion in the presence of agent for polymerisation and appropriate nucleoside triphosphates whereby amplification is effected by
5 primer extension based on the use of a single strand of sample nucleic acid as template.

An important feature of the application of PCR to this method of the present invention is the need to avoid contamination. Another important
10 feature is that the present invention requires that two rounds of PCR are effected (dual PCR amplification). This is preferably achieved using 2 pairs of nested amplification primers. It is important that the degree of specificity of the
15 primers is as high as possible. If the primers are not specific the number of cycles of PCR which can be effected without producing false positives will be reduced i.e. sensitivity will be reduced. This is particularly important when amplifying sequences
20 present in low copy numbers.

In sex linked genetic disorders serious clinical problems occurs only in males, although females may be carriers of the condition. The ability to detect male fetuses allows one to
25 advise mother bearing male fetuses to have further more invasive diagnostic tests such as chorionic villus sampling and amniocentesis. Mothers bearing female fetuses could be spared the potential dangers of such invasive procedures.

The present invention thus permits genetic
30 analysis of the foetus by the use of maternal blood without the requirement of first separating foetal and maternal cells. Thus for example the method of the present invention may be of interest in the
35 prenatal diagnosis of sex-linked conditions such as haemophilia where, if desired, chorionic villus

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sampling or amniocentesis could subsequently be performed to allow further analysis depending on the predicted sex of the foetus. It may also be possible to determine the presence in the foetus of a dominant condition carried by the father by analysis of amplified paternal derived sequences in foetal DNA. Such mutations may be detected using the amplification refractory mutation system (ARMS). The use of this amplification refractory mutation system (ARMS) as described in Nucleic Acids Research Vol. 17, No. 7 1989 p2503-2516 and as described and claimed in European Patent Publication No. 332435. The present invention may also be useful in the veterinary field where sexing of the foetus is of wide practical importance.

The method of the present invention is however of wider applicability than the prenatal diagnosis of sex-linked conditions since it is possible to selectively eliminate maternal nucleic acid from a mixture of maternal and foetal cells for example by the discriminatory use of acid or alkali media, by the use of antibodies, for example improved monoclonal antibodies capable of binding either maternal or foetal cells, by the discriminatory use of osmotic shock, by the discriminatory use of heat and/or by the discriminatory use of lysing agents such as dithiothreitol (DTT). Thus for example acid or alkali may be used to lyse maternal cells whilst leaving foetal cells intact. This may be performed by adding an ever increasing quantity of acid or alkali to a mixture of maternal and foetal cells and determining the quantity of acid or alkali necessary to lyse maternal cells whilst leaving foetal cells intact. Examples of this type of differential lysis experiment are well known, for instance in this area of forensic medicine where various concentrations of dithiothreitol. DTT may be used to effect selective

lysis of blood or sperm cells from mixtures of the two allowing DNA specimens from either cell type in the mixture to be separately prepared (Hopkins B. et. al. Technique November 1989; and Gill et. al. Nature Vol. 318, pages 577-579, 1985). These techniques may be of use in the separation of foetal cells from maternal cells or in the relative enrichment of the foetal component in a mixture of maternal and foetal cells. Where foetal cells are separated from maternal cells prenatal diagnosis of genetic disorders and predispositions other than sex-linked conditions becomes possible.

The present invention also allows an estimation to be made of the number of foetal cells per unit volume of maternal blood. By amplifying multiple aliquots from DNA samples from pregnant women, an estimation of the number of circulating foetal cells may be made. The DNA sample may either be undiluted or diluted by a known factor.

20 This approach is based on the Poisson's
distribution, which states that:

$$P(n) = \frac{e^{-m} m^n}{n!} \dots\dots\dots (1)$$

25 where n = the actual number of foetal cells
in an aliquot for PCR
 $P(n)$ = probability that there are n foetal
cells in an aliquot
30 m = mean number of foetal cells per
aliquot
 e = 2.718

In a special case of equation (1), where
35 $n=0$, i.e., when there are no foetal cells in a
particular PCR aliquot; and consequently no foetal PCR

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product, the equation becomes:

$$P(0) = \frac{e^{-m} m^0}{0!}$$

5

$$P(0) = e^{-m} \dots\dots\dots (2)$$

In practical terms, in an experiment designed to amplify foetal genetic materials, the proportion of reactions that fail to generate a signal may be approximated to $P(0)$, and using equation (2) the mean number of foetal cells in the starting DNA sample may be calculated. We emphasise that the proportion of negative reactions is only an approximation because it is known that occasional PCR may fail sporadically. For more accurate quantification of foetal cells, the proportion of sporadic PCR failure may be taken into account, e.g., by running a series of control tubes to estimate the failure rate.

We have applied this approach to six male pregnancies: three in the first trimester and three in the last trimester. The details of the cases and the results from four amplifications per case are tabulated below:

25

| CASE | GESTATION AGE (WEEKS) | NO. POSITIVES/NO. OF PCR DONE |
|------|--------------------------|-------------------------------|
|------|--------------------------|-------------------------------|

30

EARLY PREGNANCY

| | | |
|---|---|-----|
| 1 | 9 | 1/4 |
| 2 | 9 | 3/4 |
| 3 | 6 | 2/4 |

LATE PREGNANCY

35

| | | |
|---|----|-----|
| 4 | 38 | 4/4 |
| 5 | 41 | 4/4 |
| 6 | 38 | 4/4 |

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N.B. PCR is carried out using the dual amplification system with primers Y1.5/1.6 (40 cycles) followed by Y1.7/1.8 (25 cycles). (Primer sequences are given in Example 2).

5 Summing up the three early pregnancy cases, the proportion of negative results is 50%.

Thus,

$$P(0) = 0.5 = e^{-m}$$

10 $m = 0.69$

That is, there was, on average, 0.69 foetal cell per PCR aliquot. Assuming that 300 microgrammes of DNA were extracted from 5 ml of blood and that 2
15 microgrammes of DNA were used per PCR.

The total number of foetal cells per 5 ml of maternal blood

$$= 0.69 \times 300 / 2 = 104$$

20

This figure is similar to the results of Bianchi *et. al.* (Proc. Natl. Acad. Sci. 1990; 87: 3279-83) who calculated that there were 150 foetal cells per 20 ml of maternal blood. Our approach
25 differs from that of Bianchi *et. al.* who sorted the maternal blood using a fluorescence activated cell sorter using a monoclonal antibody to transferrin receptor and who subsequently quantified the amount of foetal DNA by densitometry of Southern blotted PCR
30 products. It is of interest to note that the estimate of foetal cell number by Bianchi *et. al.* (38 foetal cells per 5 ml) is less than ours (104 foetal cells per 5 ml), possibly because they were looking at a subpopulation of circulating foetal cells, i.e., those
35 that were transferrin receptor positive, whilst we were looking at any foetal DNA that might be present in maternal blood.

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It is also of interest to note that we have not been able to generate a negative PCR signal in the three late pregnancy cases in the experiment outlined above. This implies that there are more circulating foetal cells in late gestation than in early pregnancy. Thus, when used in a longitudinal study of pregnant women, our method may aid in the study of the physiology of feto-maternal cell trafficking. Using this system the number of foetal cells in maternal blood can be correlated to events in individual pregnancies e.g. the development of preeclampsia or placental insufficiency. In future, this system may be useful for the assessment of placental function.

15 BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the relative positions of the primers Y1.1, Y1.2, Y1.3 and Y1.4 and of a 102 base-pair fragment flanked by the latter pair. The primers Y1.1 and Y1.2 flank a 149 base-pair fragment of a Y-specific repeat sequence (see Kogan K. C. *et. al.* "An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences". N. Engl. J. Med. 1987; 317, 985-990).

25

The following Examples illustrate the present invention:-

Example 1

30

a) Patients

Seven to ten millilitres of blood were taken from the antecubital veins of pregnant women at various stages of gestation. Early pregnancy samples (9 to 11 weeks) were obtained from women undergoing prenatal diagnosis for genetic disorders at the A

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Bianchi Bonomi Hemophilia and Thrombosis Centre in Milan, Italy. The blood samples were taken prior to chorionic villus sampling and the sex as determined by PCR was compared with that obtained from cytogenetic analysis after chorionic villus culture. Late pregnancy samples (32-41 weeks) were obtained with informed consent from women attending an antenatal clinic at the Maternity Department, John Radcliffe Hospital, Oxford. The foetal sex as predicted by PCR was compared to the sex of the baby at delivery.

b) DNA Amplification

DNA was extracted from the blood samples using standard techniques (Maniatis T. *et. al.*: A Laboratory Manual. New York: Cold Spring Harbor Laboratory, 1982) except that it was performed under Class II containment conditions. Prior to sampling for PCR, each DNA sample was vortexed for 30 seconds in order to achieve partial shearing (fragmentation) of long strands of DNA and to allow DNA from the rare foetal cells to mix thoroughly with maternal DNA.

Two microgrammes of DNA were subjected to 40 cycles of PCR using primers Y1.1 and Y1.2 flanking a 149 base-pair fragment of Y-specific repeat sequence. (Kogan K. C. *et. al.* An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. M. Engl. J. Med. 1987; 317: 985-90). Each cycle involved thermal denaturation at 95°C for 75 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 150 seconds. After 40 cycles, 2µl of the PCR product solution was re-amplified with fresh reagents using primers Y1.3 d(ATTACACTACATTCCTTCCA) and Y1.4 d(AGTGAAATTGTATGCAGTAGA) bordering a 102 base-pair fragment internal to Y1.1 and Y1.2. Y1.3 and Y1.4 were designed from sequence data. (Nakahori Y. *et. al.* A human Y-chromosome specific repeated DNA family (DYZ1)a consists of a tandem array

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of pentanucleotides, Nucleic Acids Res. 1986; 14: 7569-80). The relative positions of these primers are illustrated in Figure 1. Fifteen to twenty cycles were performed for this second amplification step using the same cycling parameters as above. Ten μ l of PCR product were then analysed on an ethidium bromide stained agarose gel. Reagents, including the Taq DNA polymerase (5 units per 100 μ l reaction) were obtained from a Perkin-Elmer Cetus GeneAmp DNA Amplification Reagent Kit. Thermal cycling was performed using a Hybaid Intelligent Heating Block.

c) Precautions Against Contamination

The dual amplification system compounds the risk of detecting contamination. In addition to following all the guidelines previously described for minimising false-positive results, (Lo Y. *et. al.* False-positive results and the polymerase chain reaction. Lancet 1988; ii: 679 and Kwok S. Higuchi R. Avoiding false positives with PCR. Nature 1989; 339: 237-38 PCR reagents (including the Taq polymerase) were also routinely incubated with EcoRI (30 units per 100 μ l PCR reagents) for 2 hours at 37 $^{\circ}$ just prior to amplification. EcoRI was chosen as it cleaves inside the 149 base-pair region delineated by primers Y1.1 and Y1.2. The restriction enzyme was then destroyed by heating at 94 $^{\circ}$ C for 10 minutes before the addition of DNA samples. As a further precaution, no DNA samples from male individuals were extracted whilst these experiments were in progress and the blood samples were taken and subsequently handled only by female operators. The chances of contamination were reduced to a minimum by taking all these measures.

35 d) Results

Ten-fold serial dilutions of male DNA in female DNA were initially performed for determining

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the sensitivity of the dual nested amplification system. After the first round of amplification positive bands were present at dilutions of between 10^{-4} and 10^{-5} . However, after the second round of PCR with internal primers Y1.3 and Y1.4 (15 cycles), the detection limit was extended to 10^{-7} .

In order to obtain a system with maximum sensitivity and specificity, we varied the number of cycles used in the second round of amplification. The results are shown in Table 1. Following internal nested amplification of 17 cycles for the late pregnancy samples and 20 cycles for the early ones, we obtained correct prenatal sex determination in all cases tested. Thus for these particular primer pairs it is preferable that in the second round of amplification 15 to 20 cycles, especially between 17 and 20 cycles are employed. This "window" was found to give maximum sensitivity and specificity. The single positive case in a non-pregnant woman was obtained from a sample extracted prior to the institution of the stringent anti-contamination protocols. This serves to illustrate the importance of great care in preparing samples for PCR. Indeed it should be noted that false-positives have still sometimes been obtained at cycles of secondary nested internal amplification greater than those listed. However, this can be compared with the results obtained by Handyside *et. al.* (Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet 1989; i: 347-49) who, using a single round of 60 cycles of PCR amplification with primers Y1.1 and Y1.2 observed a faint band of PCR product in some of their female samples. It is believed this effect may have been due to some degree of autosomal cross-reactivity.

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TABLE 1 : Amplification of Y-specific sequences from maternal blood using different cycle combinations.

| Cycle Combination External & Internal | Proportion of Cases PCR-positive for Y-sequences | |
|--|--|--|
| | Non-pregnant women | Mothers with female foetuses Mothers with male foetuses |
| EARLY PREGNANCY (9-11 WEEKS) | | |
| 40+0 | 0/1 | 0/6 |
| 40+15 | 0/1 | 2/6 |
| 40+17 | 0/1 | 2/6 |
| 40+20 | 0/1 | 6/6 |
| LATE PREGNANCY (32-41 WEEKS) | | |
| 40+0 | 0/2 | 0/6 |
| 40+15 | 0/2 | 2/6 |
| 40+15* | 0/2 | 2/6 |
| 40+17 | 0/2 | 6/6 |
| 40+17* | 1/2+ | 6/6 |

* = Repeat experiments

+ = DNA not extracted under Class II containment conditions.

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Example 2

This Example shows the amplification of a single-copy Y-specific sequence from peripheral blood DNA extracted from pregnant women. This sequence constitutes part of a gene which is expressed in testicular tissue (Arnemann *et. al.* Nucleic Acids Res. 1987; 15: 8713-24). The single-copy nature of the sequence chosen was based on Southern blotting (Arnemann *et. al.*) and co-amplification experiments, in which both the Y-sequence and alpha-1 antitrypsin sequence were amplified to the same extent (Lawler *et. al.* B. R. J. Haematology 1989; 73: 205-10). The new primer sequences are

Y1.5: CTAGACCGCAGAGGCGCCAT
Y1.6: TAGTACCCACGCCTGCTCCGG
Y1.7: CATCCAGAGCGTCCCTGGCTT and
Y1.8: CTTTCCACAGCCACATTTGTC.

Y1.5 and Y1.6 are the external pair of primers and have been used by Lawler *et. al.* previously to amplify a 239 base pairs Y-specific fragment. Y1.7 and Y1.8 flank a 198 base pairs sequence internal to Y1.5 and Y1.6 and were designed from published sequence (Arnemann *et. al.*).

The dual amplification procedure was described above except that the cycle combination used was 40 cycles (with primers Y1.5/Y1.6) plus 25 cycles (with primers Y1.7/Y1.8). Initial decontamination of the reagents was done by incubating the reagents with Hinf I (30 units/100µl PCR reagent at 37°C for 1 hour). With the primer combination of Example 1 (Y1.1/Y1.2 followed by Y1.3/Y1.4) amplification above 40 cycles plus 20 cycles consistently resulted in false-positive results whereas this phenomenon is no longer seen with the new primers. Thus the problem of a narrow window of specific amplification is overcome by the new primer selection. This finding also implies

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that the suggestion by Nakagome *et. al.* (Lancet 1990; 335:291) as regard to the autosomal cross-reactivity of the previous Y-primers is correct.

We have used this system on a series of 37
5 pregnant women, with gestational ages ranging from 6
to 41 weeks (see Table 2). Early pregnancy samples (6-
11 weeks) were obtained from women under investigation
for prenatal diagnosis of possible genetic disorders
at the University of Milan. Blood samples were taken
10 before chorionic villus biopsy and the sex of the
foetus was determined from cytogenic analysis of
chorionic villus culture. Intermediate and late
pregnancy samples (16-41 weeks) were obtained, with
informed consent, from women attending an antenatal
15 clinic at the John Radcliffe Hospital, Oxford; foetal
sex was noted at delivery.

Of the 19 cases which gave rise to a
positive PCR signal, 16 women had male foetuses, with
3 false-positives (cases 20, 21 and 28), showing that a
20 single-copy foetal sequence can be amplified from
maternal peripheral blood. This is the first report
showing that the method has potential for a wide range
of single-gene disorders. Failure to generate a
positive signal in 6 women bearing male foetuses
25 (cases 3, 5, 15, 19, 31 and 37) may be explained by
sampling error. This may be overcome by amplifying
multiple aliquots from each patient. The existence of
false-positive results may be overcome by a more
stringent anti-contamination regime.

30

35

TABLE 2: PCR AMPLIFICATION OF A SINGLE-COPY Y-SPECIFIC SEQUENCE FROM MATERNAL BLOOD.

| | Case No. | Gestation Age (weeks) | PCR Signal | Sex on cytogenetics or on delivery |
|----|--|--------------------------|------------|---------------------------------------|
| 5 | | | | |
| | Early pregnancy samples | | | |
| 10 | 1 | 6 | + | M |
| | 2 | 9 | - | F |
| | 3 | 9 | - | M |
| | 4 | 9 | + | M |
| | 5 | 9 | - | M |
| | 6 | 9 | - | F |
| | 7 | 9 | + | M |
| 15 | 8 | 10 | + | M |
| | 9 | 10 | - | F |
| | 10 | 10 | + | M |
| | 11 | 11 | - | F |
| | 12 | 11 | - | F |
| | Intermediate to Late pregnancy samples | | | |
| 20 | 13 | 16 | + | M |
| | 14 | 17 | + | M |
| | 15 | 18 | - | M |
| | 16 | 32 | + | M |
| | 17 | 33 | - | F |
| | 18 | 35 | + | M |
| | 19 | 35 | - | M |
| 25 | 20 | 35 | + | F |
| | 21 | 37 | + | F |
| | 22 | 37 | + | M |
| | 23 | 37 | - | F |
| | 24 | 38 | + | M |
| | 25 | 39 | + | M |
| | 26 | 41 | - | F |
| 30 | 27 | 41 | + | M |
| 35 | | | | |

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